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Bactericidal Efficacy of Carbon Dioxide Laser Against Bacteria-Contaminated Titanium Implant and Subsequent Cellular Adhesion to Irradiated Area

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Background and Objective: **The aim of this study was to assess CO_2 laser ability to eliminate bacteria from titanium implant surfaces. The changes of the surface structure, the rise in temperature, and the damage of connective tissue cells after laser irradiation were also considered.**
Study Design/Materials and Methods: Streptococcus sanguis **and *Porphyromonas gingivalis* on titanium discs were irradiated by an expanded beam of CO_2 laser. Surface alteration was observed by a light, and a scanning electron, microscope. Temperature was measured with a thermograph. Damage of fibroblastic (L-929) and osteoblastic (MC3T3-E1) cells outside the irradiation spot and adhesion of the cells to the irradiated area were also estimated.**
Results: **All the organisms (10^8) of *S. sanguis* and *P. gingivalis* were killed by the irradiation at 286 J/cm^2 and 245 J/cm^2, respectively. Furthermore, laser irradiation did not cause surface alteration, rise of temperature, serious damage of connective tissue cells located outside the irradiation spot, or inhibition of cell adhesion to the irradiated area.**
Conclusion: **CO_2 laser irradiation with expanded beam may be useful in removing bacterial contaminants from implant surface. Lasers Surg. Med. 23:299–309, 1998.**

Key words: **cell, cultured; fluorescent dye; laser scanning microscope; peri-implantitis**

INTRODUCTION

Dental implants have been successfully used for replacement of missing teeth [1–3]. However, inflammation and infection around implants, peri-implantitis [4,5], may occur in some cases, if bacteria invade along the implant surfaces. Peri-implantitis, once arising, seems to advance more rapidly compared with periodontitis [6–8] because of the lack of fibrous attachment between implant and alveolar bone [9,10]. Elimination of bacteria from implant surfaces is thus required to treat peri-implantitis.

Some osseointegrated titanium implants, plasma-spray-coated or sandblasted ones for instance, are designed to have a rough surface to increase areas of attached surface and anchorage force in alveolar bone [11]. However, the roughness makes it difficult to eliminate bacteria from the surface by mechanical ways such as scaling [12]. CO_2 laser irradiation may be a useful way to eliminate bacteria from titanium implant surfaces because its bactericidal efficacy has been re-

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ported [13–15]. Since a CO_2 laser is reflected by a metallic surface [16], the beam may reach all facets of the rough surface of titanium implants. A CO_2 laser wave is absorbed well by water, including bacterial intracellular water [17], suggesting that irradiated bacteria on the implant surface are expected to be killed. The temperature of an irradiated implant is not expected to rise because the beam is not absorbed by the metallic surface. If this hypothesis is true, connective tissues, which are in contact with the implant, would not be damaged by CO_2 laser irradiation.

However there are no reports on the bactericidal efficacy of laser irradiation against bacteria on titanium implants with one exception [18] in which only a slight bactericidal efficacy of Nd:YAG laser was demonstrated. The aim of this study was to assess the ability CO_2 laser to eliminate bacteria from titanium implant surfaces. The changes of the surface structure, the rise in temperature, and the damage of connective tissue cells after laser irradiation were also considered.

MATERIALS AND METHODS

Laser Irradiation

Carbon dioxide laser (NIIC-15, NIIC, Japan) with a wavelength of 10.6 μm was used. The power output was 5 W, and 0.8 mW helium-neon laser beam was used as an aiming beam. To obtain a wider and more efficient irradiation area, the spot size of the laser beam was expanded to 3.95 mm or otherwise as indicated elsewhere. The other conditions are as follows: irradiation time ranged from 3 to 8 sec, energy from 15 to 40 J, and fluence from 122 to 327 J/cm^2.

Preparation of Titanium Discs

Titanium discs (8 mm × 2 mm thickness; Kobe Seiko, Japan) with the original cut surface (Machined-Ti discs), and those sandblasted by 50 μm Al_2O_3 particles (Sandblasted-Ti discs) were used. The discs were ultrasonically cleaned in acetone and then distilled water, and autoclaved at 121°C for 15 min. Before and after laser irradiation under conditions described above, changes of the titanium disc surface structure were examined by a light microscope (Olympus, Japan) and a scanning electron microscope (SEM: S430, Hitachi, Japan) at 25 kV, after gold sputter coating.

Bacterial Preparation

Microorganisms of *Streptococcus sanguis* ATCC 10556 and *Porphyromonas gingivalis* 381 were cultured in Brain Heart Infusion (BHI) broth for 7 hr and 20 hr, respectively, and harvested. After washing by centrifugation twice, they were suspended in sterile 40 mM sodium phosphate buffer (pH 7.0) to make a concentrations of 1.0×10^{10} cells/ml.

Culture Conditions for Fibroblastic and Osteoblastic Cells

Established cell lines of mouse fibroblast L-929, and mouse osteoblast MC3T3-E1 established by Kodama et al. [19,20] were used. Cells of L-929 were grown in Eagle's minimum essential medium (Dainippon Pharmaceutical, Japan) supplemented with 10% fetal calf serum (FCS), and those of MC3T3-E1 in alpha modification of Eagle's minimum essential medium (Dainippon Pharmaceutical, Japan) supplemented with 10% FCS, 0.2 mg/ml kanamycin sulfate (Meiji Seika, Japan), 5.9 mg/ml HEPES (Dojin, Japan), 2.2 mg/ml $NaHCO_3$, at 37°C under 5% CO_2 in air. For irradiation experiments, cells were suspended at the density of 1.0×10^5 cells/ml.

Bactericidal Efficacy

An aliquot of bacterial suspension (10 μl: 10^8 organisms) was placed on the surface of titanium discs to form a droplet within the irradiation spot. After laser irradiation under the conditions described above, bacteria were recovered and suspended in 2 ml of the phosphate buffer using a Vortex mixer for 20 sec. After serial ten-fold dilutions, aliquots of each dilution (100 μl) were spread on BHI-blood agar plates. Colony forming units were counted after 48 hr incubation. A total number of collected bacteria from control samples without laser irradiation was also estimated and the bactericidal efficacy of laser irradiation was calculated.

Temperature Measurement

Temperature of the titanium surfaces was measured with a Thermograph (TVS-2000, Nippon Abionics, Japan) before irradiation and immediately irradiation, 10, 20, 30, and 40 sec after. The room temperature was isothermally kept at 23°C. The irradiation was started after the temperature of the disc reached 23°C.

Survival of Fibroblastic and Osteoblastic Cells Outside the Irradiation Spot

Fibroblastic (L-929) and osteoblastic (MC3T3-E1) cells were seeded on the discs in 24-well plates at the final concentration of 1.0×10^5

cell/well and cultured for 72 hr. The cells adhered to the titanium discs were irradiated by CO_2 laser. Cells located inside the spot were covered by nail varnish to exclude them from counting. Thereafter, cells outside the spot were collected, in order to estimate the cell-damage outside the irradiation spot. Dead cells were differentiated from living cells by trypan blue staining and both were counted, independently. The survival rate was calculated as the percentage of living cells in total cells.

Dead and living cells, stained by specific fluorescent dyes, were also observed in situ by a confocal laser scanning microscope (CLSM: ACAS, Meridian, MI). Propidium Iodide (PI) was used for dead cells and acetoxymethyl-calcein (calcein-AM) for living cells [21,22]. Cells on the discs were incubated for 15 min at 37°C in 1.5 ml phosphate buffer saline (PBS) containing PI (0.015 mg/1.5 μl H_2O) and calcein-AM (0.01 mg/1 μl dimethyl sulfoxide).

Adhesion of Fibroblastic and Osteoblastic Cells on Irradiated Area

After being irradiated and killed, bacteria in the irradiation spot on the titanium disc surface were rinsed with distilled water. The discs with the killed bacteria were placed in the wells of a 24-well plate. As a control, bacteria placed on discs were autoclaved. As another control, discs without bacteria were also used. Fibroblastic and osteoblastic cells were seeded, respectively, at the final concentration of 1.0×10^5 cells/well, and cultured for 48 hr. In order to count the cells adhered to the surface where bacteria were irradiated, the cells on the area outside the irradiation spot were covered by nail varnish to be excluded from the counting, since bacteria were only placed in the spot area. Collected cells were counted after staining by trypan blue, and the rate of living cells in all cells was calculated as the survival rate. Alkaline phosphatase (ALPase) activity of the adhered osteoblasts was measured in vitro with ρ-nitrophenylphosphate by the method of Lowry et al. [23]. Protein content was measured with Protein Assay Reagent (Pierce, Rockford, IL) by the method of Bradford [24]. ALPase activity was also assayed *in situ* by CLSM after being visualized with the fluorescent azo dye technique [25]. Briefly, adhered cells on the discs were incubated in Hank,s balanced salt solution supplemented with 1% FCS, 10 mM HEPES and 0.1% Triton X-100 and reacted for 30 min at 37C° in the presence of 4 mg Naphtol AS-BI phosphate (Sigma Chemical Co., St. Louis, MO) in 300 μl of dimethyl formamide, in 10 ml of 0.1 M Tris-HCl buffer (pH 8.2) containing 10 mg of Fast red RC salt (Sigma Chemical Co., St. Louis, MO). Hoechst 33342 (Sigma Chemical Co., St. Louis, MO) was used to stain DNA at a final concentration of 5 μg/ml.

Statistic Analysis

Data were analyzed using Student's *t*-test. Differences were considered significant if the probability was less than 5%.

RESULTS

Changes of Titanium Surface Structures

Rough surface structures, such as concentric circular traces caused by mechanical cutting (Fig. 1a) were observed by light microscopy and, between the cutting traces, more fine processes were also observed by SEM (Fig. 1b). Concavities of similar diameter were observed on the surface of Sandblasted-Ti discs by light microscopy (Fig. 2a), and more complicated rough structures in the concavities were observed by SEM (Fig. 2b). After irradiation of CO_2 laser by defocused beam (3.95 mm) at a rather low fluence (122–327 J/cm^2), titanium disc surface structures were not changed. By that reason, spot size was focused to 1.1 mm to obtain a rather high fluence. After irradiation of CO_2 laser by focused beam (1.1 mm) at a rather high fluence (2,632 J/cm^2), discoloration of titanium was not observed by light microscopy and the rough surface structures on both Machined and Sandblasted-Ti discs were not altered (Figs. 1c, 2c). The SEM observation of irradiated discs also revealed that alteration of titanium surface structures, such as micro-fracture, did not occur (Figs. 1d, 2d).

Bactericidal Efficacy

Microorganisms of *S. sanguis* and *P. gingivalis* placed on the titanium disc surface were killed by CO_2 laser irradiation (Table 1). All organisms of *S. sanguis* were killed after irradiation at 286 J/cm^2 and around 90% were killed at 163 J/cm^2, both on Machined-Ti discs and on Sandblasted-Ti discs. *P. gingivalis* were more sensitive (Student's *t*-test, $P<0.01$) to laser irradiation, and 100% of the organisms were killed after irradiation at 245 J/cm^2.

Temperature Change of Titanium Discs

Laser irradiation caused slight rises of temperature on the surface of titanium discs (Table

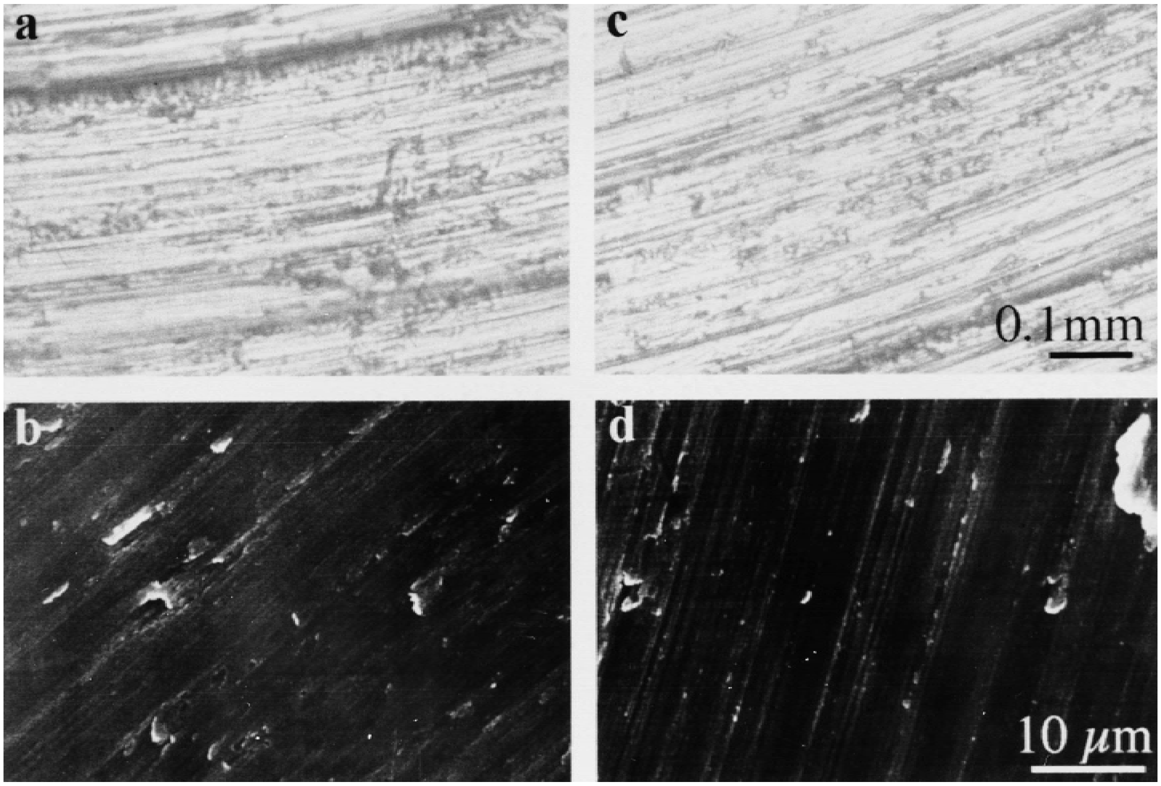

Fig. 1. **Upper:** Light microscopic observation of Machined-Ti disc (**a,c:** Original magnification, ×125). **Lower:** SEM observation (**b,d:** Original magnification, ×2000). a,b: Not irradiated; c,d: irradiated at 2,632 J/cm^2.

2). The temperature rose proportionally with increase of irradiation energy from 15–40 J. Though the rise was within 3°C at tested energy, Machined-Ti discs showed a significantly lesser (Student's *t*-test, $P<0.01$) rise of temperature than Sandblasted-Ti discs. When the laser energy was absorbed in 10 μl buffer solution, temperature of the buffer which on titanium disc surface rose more. At the center of irradiation spot, the temperature increased immediately about 12°C after irradiation at 30 J (fluence 245 J/cm^2) on both Machined-Ti discs and Sandblasted-Ti discs, and then fell with time and returned to the room temperature (Fig. 3). Outside the spot, at 1.5 mm away from the irradiation border, the temperature of the disc surface increased about 5°C at same irradiation energy, and then, fell with time.

Survival of Fibroblastic and Osteoblastic Cells Located Outside Irradiation Spot

Outside the irradiation spot irradiated at 245 J/cm^2, fibroblastic cells (L-929) survived 86–89%, and 75–78% at the rather high fluence of 327 J/cm^2 (Table 3). Similarly, osteoblastic cells (MC3T3-E1) survived 80–84% at irradiation of 245 J/cm^2 and 67% at 327 J/cm^2. The nail varnish did not harm the surrounding cells. CLSM observation clearly demonstrated that cells in the spot were stained by PI, suggesting they were dead and that cells outside the spot were stained by calcein-AM, suggesting they were living (the color photos are not shown). Dead cells and living cells were clearly separated at the border of irradiation area (Fig. 4).

Cellular Adhesion on Bacteria Smeared and Laser Irradiated Surface

Fibroblastic (L-929) and osteoblastic (MC3T3-E1) cells could adhere to titanium disc surfaces and the survival rates were 92–97% (Fig. 5. No-bacteria). Similarly the cells could adhere the surfaces where bacteria were smeared and killed by laser irradiating (245 and 286 J/cm^2) or autoclave. Almost all the adhered cells were vital (81–92%), indicating bacterial debris after irradiation did not effect the cellular adherence and vitality (Fig. 5). Osteoblastic cells seemed to be more affected than fibroblastic cells. ALPase ac-

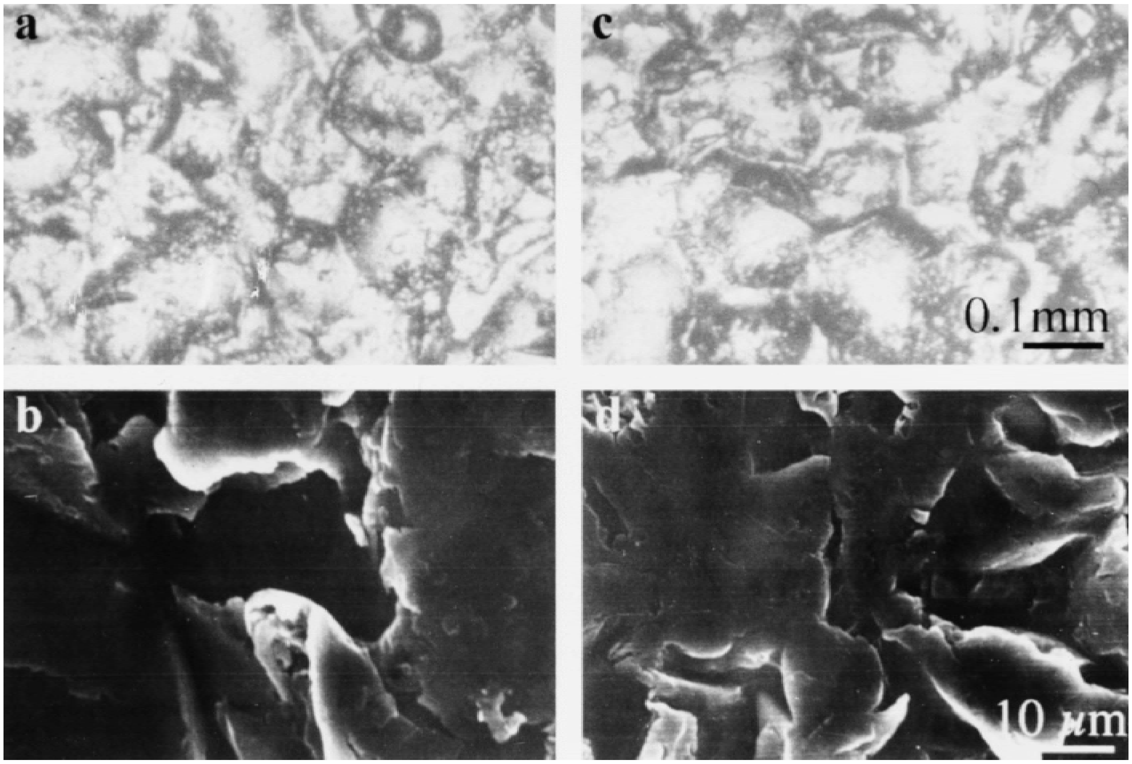

Fig. 2. Light microscopic and SEM observation of Sandblasted-Ti disc. For the symbols, see the legend in Figure 1.

TABLE 1. Bactericidal Efficacy of CO_2 Laser for Oral Bacteria on Titanium Discs*

Energy (J)	Fluence (J/cm^2)	Bacterial strain: *S. sanguis* ATCC 10556, Machined-Ti	*S. sanguis* ATCC 10556, Sandblasted-Ti	*P. gingivalis* 381, Machined-Ti	*P. gingivalis* 381, Sandblasted-Ti
		Mean kill ± S.D. (%)			
15	122	74.2 ± 8.2	80.2 ± 7.8	98.6 ± 0.6	98.7 ± 0.8
20	163	88.4 ± 6.8	89.5 ± 7.3	99.9 ± 0.1	99.8 ± 0.1
25	204	96.1 ± 2.9	94.9 ± 5.7	99.9 ± 0.0	99.9 ± 0.0
30	245	99.6 ± 0.3	99.6 ± 0.6	100 ± 0	100 ± 0
35	286	100 ± 0	100 ± 0	100 ± 0	100 ± 0
40	327	100 ± 0	100 ± 0	100 ± 0	100 ± 0

*(n = 5, *P. sanguis* or 3, *P. gingivalis*)

tivity of osteoblastic cells that adhered to the irradiated area was similar to that of cells adhered to titanium disc surfaces without bacteria (Table 4. Student's *t*-test, $P<0.01$). CLSM observation in situ revealed that osteoblastic cells showed ALPase positive regardless of location on the disc (Fig 6).

DISCUSSION

The present study clearly demonstrated that irradiation with carbon dioxide laser can efficiently kill bacteria on titanium disc surfaces.

Since bacterial infection is one of the main causes of peri-implantitis [26,27], elimination of bacteria from the surface of dental implants is required to treat, and even to prevent, peri-implantitis. Bacterial elimination may also lead to a good result of bone formation in guided bone regeneration [28–30]. To eliminate bacteria from the surfaces, mechanical instruments have been generally used. Systemic and/or local antimicrobial therapy [31–33] may also be performed to control bacterial infection and to reduce inflammation. However, some instruments, such as

TABLE 2. Temperature Rise of Titanium Discs After CO_2 Laser Irradiation*

	Center of irradiation spot			
	Wet		Dry	
Energy (J)	Machined-Ti	Sandblasted-Ti	Machined-Ti	Sandblasted-Ti
	Mean temperature change ± S.D. (°C)			
15	7.7 ± 0.8	8.2 ± 0.4	0.3 ± 0.2	1.0 ± 0.2
20	9.2 ± 0.5	9.8 ± 0.9	0.6 ± 0.2	1.2 ± 0.3
25	10.5 ± 0.3	10.7 ± 0.6	0.7 ± 0.2	1.7 ± 0.2
30	11.7 ± 0.9	12.0 ± 0.6	0.9 ± 0.1	2.0 ± 0.2
35	12.8 ± 0.7	12.9 ± 0.4	1.0 ± 0.2	2.2 ± 0.4
40	13.9 ± 0.8	13.9 ± 0.6	1.2 ± 0.2	2.7 ± 0.4
	Outside the spot			
	Wet		Dry	
Energy (J)	Machined-Ti	Sandblasted-Ti	Machined-Ti	Sandblasted-Ti
	Mean temperature change ± S.D. (°C)			
15	1.6 ± 0.3	2.3 ± 0.3	0.1 ± 0.1	0.8 ± 0.2
20	2.4 ± 0.4	3.0 ± 0.5	0.3 ± 0.1	0.9 ± 0.2
25	3.7 ± 0.3	3.9 ± 0.6	0.3 ± 0.1	1.2 ± 0.2
30	4.3 ± 0.6	4.8 ± 0.6	0.4 ± 0.1	1.4 ± 0.2
35	5.1 ± 0.5	5.4 ± 0.6	0.5 ± 0.1	1.7 ± 0.3
40	5.8 ± 0.3	5.8 ± 0.4	0.5 ± 0.1	1.9 ± 0.4

*n = 5. Wet, in presence of 10 μl buffer solution. Dry, in absence of the solution.

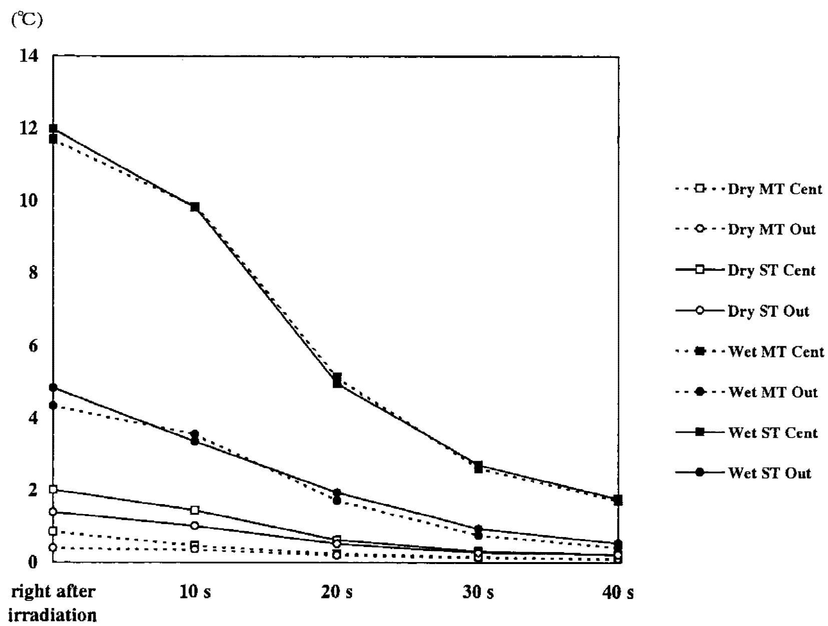

Fig. 3. Rise of temperature at the center of irradiation spot (Cent) and outside the spot (Out) on the titanium disc after irradiated at 30 J. MT: Machined-Ti disc, ST, Sandblasted-Ti disc.

various scalars [34,35] and air-powder-flow [36], may not easily eliminate bacteria from titanium implant surfaces because of its roughness. Moreover, some of these instruments have been reported to damage the titanium implant surface structure [34–36] and cause dissimilar metallic contamination [34,35] or adhesion of abrasive powder to the implants fixture [36]. Air-powder-flow can cause emphysema, demineralization of alveolar bone, and marginal bone loss around the fixture [37].

In the present study, a CO_2 laser was selected among others used for dental treatment, such as Nd:YAG or KTP:YAG, which have also

TABLE 3. Survival Rate of Fibroblastic and Osteoblastic Cells Outside the Irradiation Spot*

Fluence (J)	Fibroblastic cells		Osteoblastic cells	
	Machined-Ti	Sandblasted-Ti	Machined-Ti	Sandblasted-Ti
	Survival rate ± S.D. (%)			
0	100	100	100	100
163	90.0 ± 2.0	92.1 ± 2.5	87.6 ± 2.8	85.6 ± 3.2
245	86.8 ± 4.4	89.5 ± 3.6	84.4 ± 4.8	80.9 ± 3.1
327	75.2 ± 4.1	78.6 ± 3.8	67.6 ± 3.9	67.2 ± 3.9

*n = 7.

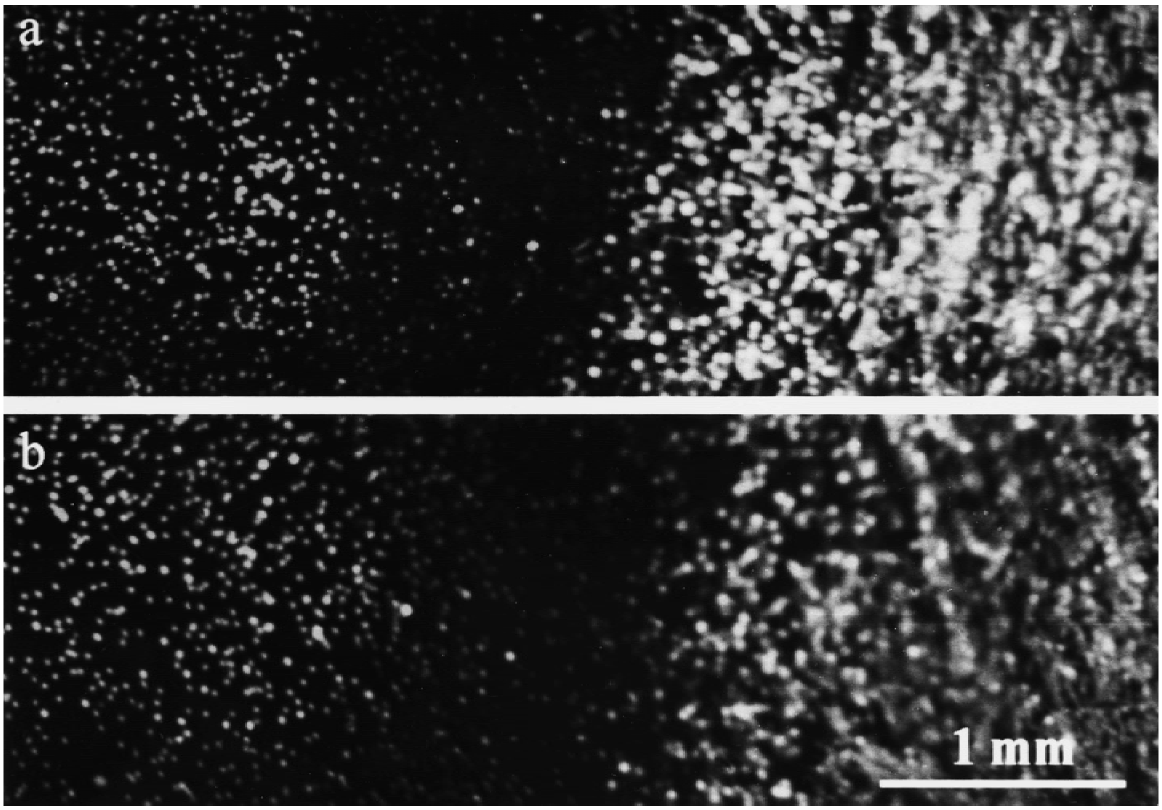

Fig. 4. Fluorescence photomicrographs of osteoblastic cells on Machined-Ti discs. Living and dead cells were stained by calcein-AM and PI, respectively. **a:** 163 J/cm^2, **b:** 245 J/cm^2.

reported bactericidal efficacy [38,39]. Preliminary experiments showed that to obtain the same bactericidal efficacy, higher energy was required in the irradiation of titanium discs by Nd:YAG and KTP:YAG lasers than by CO_2 laser. In addition to this high energy requirement, Nd:YAG and KTP:YAG lasers are short wavelength lasers, and thus the beams are better absorbed by metal than CO_2 laser of longer wavelength [12]. Results of preliminary experiments showed that the temperature of titanium discs increased 7–9 times more after irradiation by Nd:YAG and KTP:YAG lasers than by CO_2 laser.

Bactericidal efficacy of CO_2 laser have been measured on metal blades [13] and glass-slides [14] but not so far on the titanium surface. In the present study, 90% organisms of *S. sanguis* on the titanium surface were killed by CO_2 laser irradiation at 163 J/cm^2 and 100% at 286 J/cm^2 in agreement with the result by Dederich et al. [14]. They reported that 98.5% organisms of *S. sanguis* ATCC 10556 on glass slides were killed by CO_2 laser irradiation at 159 J/cm^2. Slight discrepancies between their results and those in the present study may be ascribed to the fact that the bacterial number irradiated in their study is 1/

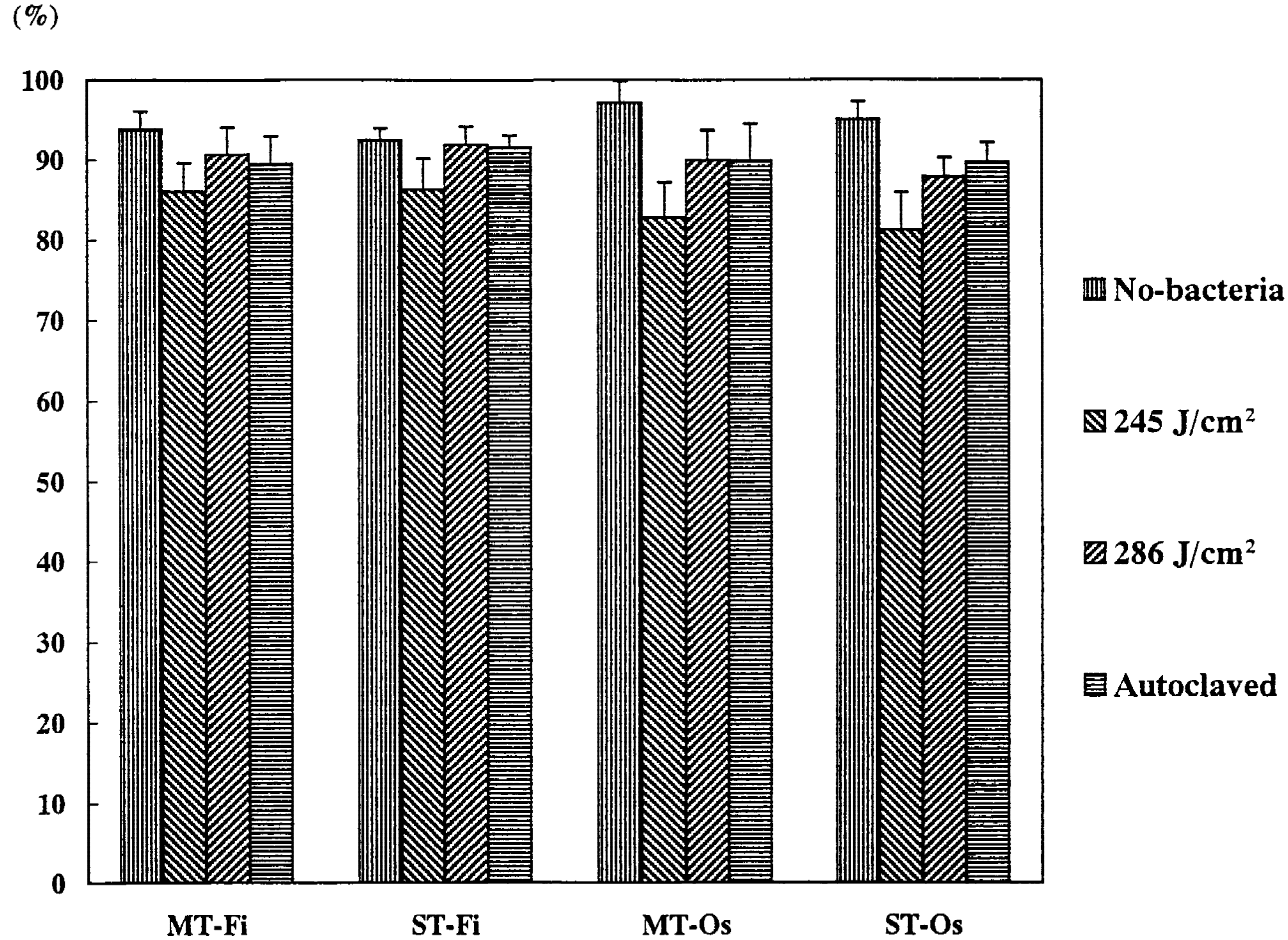

Fig. 5. Survival rate of fibroblastic and osteoblastic cells adhered on the bacteria-free titanium surfaces (No-bacteria), on the surfaces where bacteria were smeared and killed by laser irradiation (245, 286 J/cm^2) or autoclave (Autoclaved). Fi: fibroblastic cells, Os: osteoblastic cells. For other abbreviations, see in Figure 3.

TABLE 4. ALP Activity of Osteoblastic Cells* Adhered to Irradiated Area

	ALP activity (nmole/mg protein/min)	
Area	Machined-Ti	Sandblasted-Ti
245 J/cm^2	10.0 ± 1.5	9.7 ± 2.3
286 J/cm^2	10.2 ± 1.8	10.7 ± 3.1
No-bacteria	11.0 ± 2.5	11.8 ± 3.1
Autoclaved	10.5 ± 1.8	11.6 ± 2.0

*Area, Osteoblastic cells adhered at area where bacteria were irradiated at 245 or 286 J/cm^2, where bacteria were killed by autoclavity but not irradiated, and where no bacteria were placed and irradiation was not given (n = 5).

10–1/100 of the present study. The bacterial concentration in the present study was considered to be closer to that in dental plaque [40,41] or periodontal pocket fluid [42]. *P. gingivalis* was more sensitive to CO_2 laser irradiation than *S. sanguis.* The reason for this difference may be ascribed to the fact that *P. gingivalis* belongs to *Tenericutes* (i.e., Gram-negative bacteria) having a soft nature and lacking of a rigid cell wall, while *S. sanguis* belongs to *Firmicutes* (i.e., Gram-positive bacteria) having a firm nature [43]. Laser irradiation may cause decomposition and vaporization of bacterial chemical constituents by the photothermal and photoabrasive effects described previously [17].

In this study we used an expanded beam rather than a focused beam to irradiate a wider area at one irradiation time. It may be rather difficult to irradiate certain area of titanium implants with a focused laser beam to kill bacteria. Expanded beam may make it easier to irradiate titanium implant surfaces and thus save clinical time.

Outside the irradiation spot of the titanium implant, where the implant is in contact with host connective tissue, slight cell–damage by the photothermal effect may occur because CO_2 laser may be reflected by the titanium surface and

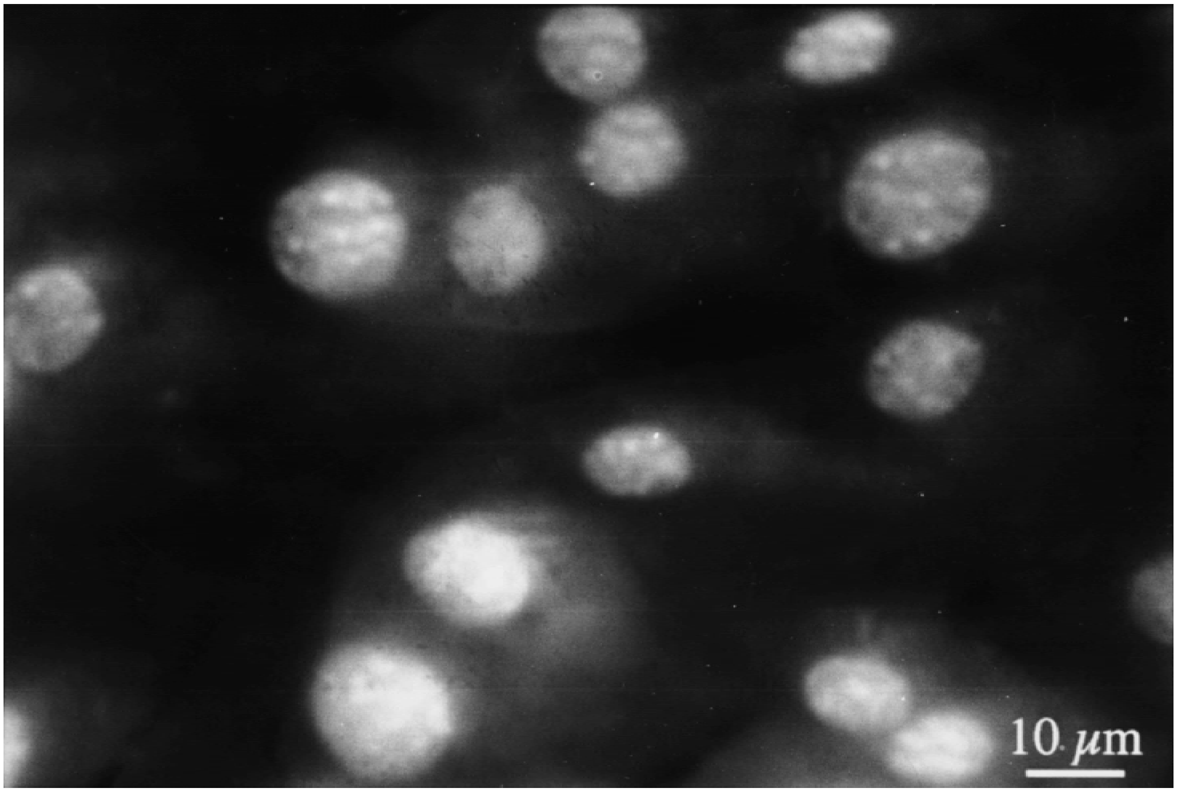

Fig. 6. Alkaline phosphatase in osteoblastic cells where bacteria were irradiated (245 J/cm^2) on Machined-Ti disc. The enzymatic activity was visualized by fluorescent azo dye technique.

reach outside the irradiation spot. However, in the present study, only a small number of fibroblastic and osteoblastic cells were damaged and almost all the cells located outside the spot were not damaged as shown by CLSM. This suggest that the rough surface of titanium disc reflects the beam inside the concavities and most cells were not irradiated fatally.

Damage of cells outside the irradiation spot may also be caused by the temperature rise of titanium implant bodies by the irradiation. It has been reported that fibroblastic cells are injured by heating to 44°C for 5 min [44] and bone tissue is injured by 47°C for 1 min [45]. However, in the present study, CO_2 laser irradiation at bactericidal dose caused only a slight rise of temperature, indicating that surrounding tissues, outside the irradiation spot of titanium implant may be safe. This minimal temperature rise would be caused because titanium reflected this laser wavelength. In vivo, however, it is afraid that the reflected energy may absorbed by adjacent tissues, and as a result injure the tissues and heat may conduct back to surrounding tissues. Thus, protection should be considered to prevent damage the tissues by mean of wet gauze and so on.

Fibroblastic and osteoblastic cells adhere to the area where bacteria were killed by CO_2 laser irradiation, suggesting that the laser irradiation may not interfere the subsequent cellular adhesion of connective tissue. A Pase activity of such adhered osteoblastic cells, monitored as an indicator of the viability, was demonstrated to be almost same to that of cells adhered to titanium disc surface without bacteria. This activity also agrees with that reported in cultured osteoblastic cells [46].

With the results of this in vitro experiment, it can be conclude that CO_2 laser irradiation with expanded beam may be useful in removing bacterial contaminants from implant surface without alteration of titanium implant surface structure, and the irradiation may not damage peripheral connective tissues and allow them to adhere to the implant. However, for the clinical application, a further study is required to establish in vivo irradiation conditions suitable to treat periimplantitis.

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